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Review

Role of immune response in *Yersinia pestis* infection

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Abstract

Yersinia pestis (*Y. Pestis*) is an infamous pathogen causing plague pandemics throughout history and is a selected agent of bioterrorism threatening public health. *Y. pestis* was first isolated by Alexandre Yersin in 1894 in Hong Kong and in the years to follow from all continents. Plague is enzootic in different rodents and their fleas in Africa, North and South America, and Asia such as Middle/Far East and ex-USSR countries.

Comprehending the multifaceted interaction between *Y. pestis* and the host immune system will enable us design more effective vaccines.

Innate immune response and both component (humoral and cellular) of adaptive immune response contribute to host defense against *Y.pestis* infection, but the bacterium possess different mechanisms to counteract the immune response.

The aims of this review are to analyze the role of immune response versus *Yersinia pestis* infection and to highlight the various stratagems adopted by *Y. pestis* to escape the immunological defenses.

Key words: *Yersinia pestis*; innate immunity; adaptive immunity.

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Introduction

Yersinia pestis was first isolated by Alexandre Yersin in 1894 in Hong Kong [1] and the role of fleas in the transmission of plague was subsequently identified [2]. *Y. pestis* has been isolated in all continents, and plague is enzootic in various rodents and their fleas in Africa, North and South America, and Asia such as Middle/Far East and ex-USSR countries [3,4].

Plague is seasonal in most endemic countries with a well defined geographical distribution, which correlates with that of the predominant vectors and rodent reservoirs and their ecology [5].

In 2000 and 2001 more than 95% of reported human cases were from the African region, including a well documented focus in Madagascar accounting for more than the 41% of the world's reported cases [6]. Bubonic plague is the predominant form reported worldwide (80–95% of cases) [7], with a mortality rate of 10–20% [6,8].

Increased mortality (22%) is seen in a small proportion of patients (10–20%) who develops systemic *Yersinia pestis* sepsis without bubo (primary septicaemic plague) [8].

Another rare disease form is primary pulmonary plague which has a mortality rate of 100% if untreated and more than 50% with antimicrobial treatment [7,9].

Yersinia pestis is a Gram-negative, non-motile, facultative intracellular bacterium [4] and multilocus sequence typing of housekeeping genes suggests that *Y. pestis* is a clone derived from *Yersinia pseudotuberculosis*, an enteric pathogen. The annotated genome sequences of five strains of *Yersinia pestis,* and one strain of *Yersinia pseudotuberculosis* have been reported, and they show a substantial conservation of DNA sequence and gene complement between *Y. pestis* and *Y. pseudotuberculosis* [10].

Like its cousins, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, *Y. pestis* is host to the plasmid pCD1. In addition, it also hosts two other plasmids, pPCP1 (also called pPla or pPst) and pMT1 (also called pFra) which are not carried by the other *Yersinia* species. pFra codes for a phospholipase D that is important for the ability of *Yersinia pestis* to be transmitted by fleas [11]. pPla codes for a protease, Pla, that activates plasminogen in human hosts and it is a very important virulence factor for pneumonic plague [12]. Together, these plasmids, and a pathogenicity island called HPI, encode several proteins which cause the pathogenesis, which *Yersinia pestis* is famous for.

Among other things, these virulence factors are required for bacterial adhesion and injection of proteins into the host cell, invasion of bacteria in the host cell (via a Type III Secretion System), and acquisition and binding of iron that is harvested from red blood cells (via siderophores).

Wild rodent populations are the primary natural reservoirs for *Y. pestis* [13]. In that setting, blood feeding ectoparasites, primarily fleas, transmit the bacilli from one rodent to another and from rodent to humans (bubonic plague) [14].

Recently *Yersinia pestis* is classified as a bioterrorism agent [15] but it already has a long history as a biowarfare agent [16]. Despite signing the Biological Weapons Convention in 1972, Soviet scientists developed the requisite technology to deploy large quantities of aerosolized *Yersinia pestis* [16]. Moreover, antibiotic-resistant *Yersinia pestis* strains are now known to exist [17] and aerosolized would be a formidable and also an intimidating weapon of terror [16].

In humans, the defense against pathogens rests on the early responses mediated by innate immunity and on the delayed responses of the specific immunity. The innate immunity consists of mechanisms, able of a rapid reaction against microbes and it is composed by: physical / chemical barriers (i.e. epithelium and antimicrobial substances); phagocytic cells (i.e. macrophages, neutrophils, and natural killer (NK) cells) and soluble mediators (such as complement proteins, cytokines, chemokines). In detail, the epithelial tissues act as physical and functional barriers to the infectious agents, preventing their access and their growth through the production of natural antimicrobial substances. If these barriers are overcome, microbes face up to professional phagocytes, in particular neutrophils and macrophages, which are able to kill them by their incorporation and digestion in an intracellular compartment called phagolysosome. Macrophages also secrete cytokines and chemokines that stimulate the inflammatory process and lymphocyte responses.

Finally, soluble mediators, such as complement proteins activated by the alternative pathway, are able to eliminate microbes directly by lysis or through their opsonization, so favoring their phagocytosis and subsequent killing.

The exposure to an infectious agent puts into motion also advanced mechanisms, which need more time to be established (four / five days), known as adaptive immune responses. The adaptive (or specific) immunity, mediated by T and B lymphocytes, provides a specific protection to the host and a long-lasting immunological memory. Lymphocytes trigger specific immune responses due to the expression of a highly diversified and clonally distributed repertoire of receptors, which interacts with foreign antigens. Any specific response begins in peripheral lymphoid tissues, such as lymph nodes, spleen, and mucosa-associated lymphoid tissues. Here, the naive lymphocytes recognize the antigen presented on the surface of the professional "antigen presenting cells" (APC), in combination with major histocompatibility complex (MHC) molecules. The antigen recognition and the following lymphocytes' activation determines the generation of effector and memory cells. B-lymphocytes represent the main defense against extracellular microbes and their toxins, through the release of antibodies in the so-called humoral immunity. Instead, the different T lymphocytes defend against intracellular microbes, viruses and bacteria, favoring the destruction of the microbes incorporated in the phagocytes or the lysis of infected cells.

Important features of both the T cell-mediated and the humoral responses are specificity, diversity, memory, specialization, self-limitation, and discrimination of self. Overall, the host is able to eradicate the infections only thanks to the coordinated action of the innate and adaptive immune responses [4].

Anyway, pathogens may develop stratagems to evade the innate and the immune adaptive responses; for example, *Y. pestis* uses several tricks to avoid the immune system control.

This review focuses on the role of the immune response versus *Yersinia pestis* infection and on the analysis of the various mechanisms adopted by the bacterium to escape the immunological defenses.

Evasion of innate host responses

Yersinia pestis, through the bite of an infected flea, directly exceeds the skin barrier and reaches the phagocytes at the invasion site. Even if the majority of the bacilli can be eliminated by neutrophils, the facultative intracellular *Yersinia pestis* infects macrophages, by the recognition of specific surface-associated CCR5 molecule [18], where it proliferates and express virulence determinants.

After the acquisition of a phagocytosis resistance profile, it can be delivered from the infected cell to the extracellular space and then, it can propagate into the circulatory system [18]. In this context, *Yersinia pestis* may evade the host innate immune system both at the early stage of infection and after the release of *Y. pestis* from macrophages. First, the Lipopolysaccharides (LPS) structure, during the passage from gut flea to the

host temperatures changes, making *Yersinia pestis* resistant to the serum-mediated lysis and repress the inflammatory response (Figure 1A).
Second, the bacilli escaped from macrophages acquire resistance to phagocytosis and are able to abate the production of pro-inflammatory cytokines and chemokines (Figure1B).

Inhibition of TLR4-mediated activation and resistance to complement-mediated lysis

LPS are large molecules consisting of a lipid A and a polysaccharide composed of O-antigen, localized in the outer leaflet of Gram-negative bacteria. LPS are recognized by the Toll-like receptor (TLR) 4 whose engagement initiates immune responses [19]. Anyway, the TRL4 signaling is affected by the fatty acid side chains' composition of LPS lipid A. The maximum stimulation is obtained by hexa-acylated lipid A with side chains by 12 to 14 carbons while changes in the number or length of the attached fatty acids decreases the signal strength [20].

Several studies demonstrated that *Y. pestis* synthesizes different forms of LPS depending on host-specific environmental conditions [21]. For example, Lien's group has demonstrated that *Y. pestis* contain a mixture of stimulatory and non-stimulatory LPS, particularly during the passage from flea to the host [22]. In fact, the temperature differences from the gut flea (26°C) to the human host (37°C) favor the synthesis of a tetra-acylated form of LPS, that unlike the typical hexa-acylated LPS, it is not able to induce the TLR4-mediated production of pro-inflammatory cytokines (IL-1, IL-6, TNF-α, IL-8) [22]. In this way, *Y. pestis* may efficiently block macrophages' activation and secretion of cytokines, which in turn prevents further activation of dendritic cells, essential for the induction of adaptive immune response [22].

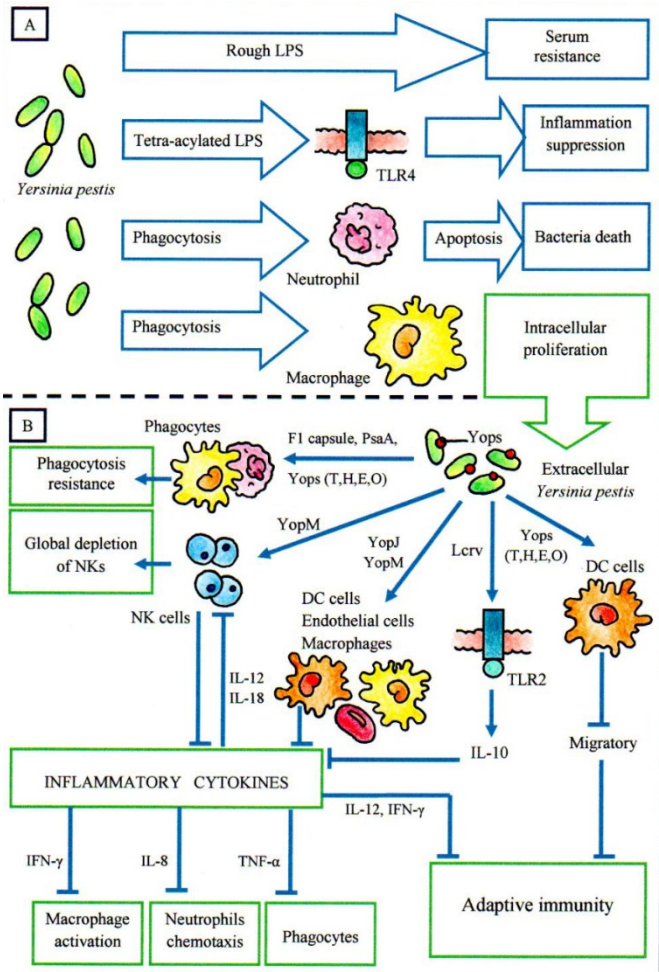
In addition, *Y. pestis* has evolved the ability to resist to complement dependent killing in order to survive in the circulation of human host [23-25]. *Y. pestis*, compared to other Yersinia spp. (i.e. *enterocolitica*), is able to survive to the complement's action either at 25°C or 37°C [24]. In enteropathogenic yersiniae, this function is rather mediated by the outer membrane proteins YadA. Otherwise, in *Y. pestis* that do not express YadA, the serum resistance seems to be mediated by LPS and Ail [25]. In fact, Plano and colleagues have widely demonstrated the Ail's mediated protection of *Y. pestis* from *in vitro* complement dependent killing [25].

Acquirement of phagocytosis resistance

Phagocytosis is an essential protective mechanism of the innate immunity, mediated by cells able to ingest and destroy microorganisms. In detail, phagocytes ingest the pathogenic microorganisms, and trap them in a phagosome, which then fuses with a lysosome to form a phagolysosome. Within the phagolysosome, enzymes and toxic peroxides digest the pathogen.

As soon as *Y. pestis* overcomes the mucosal barriers, it is phagocytosed by competent cells, and the neutrophils, in the first two days post infection, confine the *Y. pestis*' spread [26]. Anyway, *Y. pestis*, through specific surface-associated molecules, infects the macrophages that appear to be more "compliant" than

Figure 1. Mechanisms of *Y. pestis'* resistance to host innate immunity.



A) Changes in the Lipopolysaccharides' (LPS) structure, during the passage from gut flea to host temperatures, making *Y. pestis* resistant to the serum-mediated lysis. B) The bacteria released from macrophages acquire resistance to phagocytosis and they are able to abate the production of pro-inflammatory cytokines.

neutrophils, allowing the surviving and the intracellular growth of plague bacilli. This mechanism is indispensable for the plague pathogenesis: it give protection to the bacteria avoiding the contact with other immune components; it provides a replicative niche in which the bacilli can become conditioned to growth at 37°C; it supplies the time for the expression of virulence determinants that make *Y. pestis* resistant to phagocitosis [27]. Finally, it provides a transport vehicle to the local draining lymph node [28]. After four and five days from infection, the *Y. pestis* bacilli can get away from macrophages spreading into the extracellular space with phagocytosis resistance and causing bacteriemia.

The work of Oyston and collaborators has interestingly shown the possible role of PhoP/PhoQ in mediating the intracellular *Y. pestis* survival in macrophages [29].

After one to four hour of macrophages' infection, the plague bacillum increases the expression of virulence markers such as Yops, F1 antigen, and V antigen [4]. In particular, the expression of F1 antigen permits the formation of a capsule around the bacterium, that protects bacteria from the phagocytosis. F1 seems to interfere at the level of receptor interaction with macrophages and neutrophils working with a different mechanism from that of the type III secretion system (T3SS) [30].

The determinant pH 6 antigen (PsaA) of *Y. pestis* is expressed in response to environmental factor, such as temperature above 34°C and low pH, similar to the macrophages phagolysosome [31]. Recently, a study reported that purified PsaA selectively bounds to apolipoprotein B (*apoB*)-containing lipoproteins in human plasma [32]. The binding of LDL to the bacterial surface could prevent recognition of the pathogen by the macrophages, favoring the establishment of *Y. pestis* infection [32].

Furthermore**,** the T3SS genes, located in a 70 kb virulence plasmid, are a key factor to allow the systemic spreading of plague bacilli. Inside the macrophages, at the temperature of 37°C, T3SS genes are expressed leading to the formation of an injectisome on the surface of the bacillum [33]. Then, after the evasion from macrophages, *Y. pestis* upon contact with immune cells (macrophages, DCs, and neutrophils), inject in their cytoplasm six different Yersinia outer proteins (Yops; YopE, YopJ/YopP, YopM, YopH, YopT, and YpkA/YopO) thus inhibiting the host immune responses [34].

Negative effects on the innate cellular district

The natural killer cells are a lymphocytes' subset of cells able to kill infected cells by lysis, without the antigens recognition, and to secrete cytokines, in particular IFN-γ, enhancing the phagocytic activity of macrophages. Evident yet at the second infection day, *Y. pestis*, through the effector YopM, cause a high decrease in the NK cells' number, resulting in a poor secretion of IFN-γ that decreases the synthesis of reactive nitrogen intermediates by macrophages [35].

The T3SS proteins YopE, YopT, and YopO, can interfer with the host cell actin regulation of Rho GTPases, inhibiting the *Y. pestis* phagocytosis. Moreover, YopH can inactivate host proteins associated with signaling from the receptor to actin and directly suppress the production of intracellular ROS by phagocytes [36-38]. In addition, the effector protein YopM (an important virulence factor in Yersinia infection in mice) [39] migrates to the nucleus by means of a vesicle-associated pathway [40] thus affecting the expression of genes involved in cell cycle and cell growth, by direct interaction with the protein kinase C-like 2 and ribosomal protein S6 kinase [41].

Yops are also able to inhibit the production of inflammatory cytokines by infected cells; for example, YopPs inhibit the production by macrophages and endothelial cells of TNF-α the most important cytokine to counteract bacterial infection, and IL-8 [42].

An additional *Y. pestis* protein implicated in the inhibition of proinflammatory factors' production is LcrV (low-calcium-response V or V antigen), a multifunctional protein involved in contact-induced secretion of Yops' proteins [43]. Once released in the extracellular space, LcrV leads to immunosuppression by a TLR2/CD14- dependent signaling that induces the production of IL-10 and suppresses the TNF-α and IFN-γ release [44]. The LcrV immunomodulatory properties are demonstrated by the fact that an LcrV mutant strain lacking short amino acid residues (residues 271 to 300) protected animals from *Y. pestis* infection eliciting effector immune responses [45].

Other mechanisms used by *Y. pestis* can disturb the host immune responses; for example, the effectors YopP/YopJ once pumped in the endothelial cells decrease the expression of adhesion molecules (ICAM-1 and E-selectin) on endothelial and bronchial epithelial cells, thus inhibiting the recruitment of polimorphonuclear cells to the infection site [46,47]. Moreover, LcrV can inhibit the neutrophil chemotaxis both *in vivo* and *in vitro* [48].

Furthermore, the suppression of proinflammatory factors' production not only reduces the activation of

innate immune cells, but also compromises the inflammatory environment that is essential for the adaptive immune response.

Escape of adaptive host immune response

The adaptive immune responses are carried on by T and B cells, activated by the pathogen-associated antigens recognition and the costimuli received by the innate immune cells. It is characterized by antigen-specificity and long-term immunological memory. The plague bacilli are able to reduce the host adaptive immune response influencing the cytokine/chemokine induction (discussed in section 1 and in Figure 1) as well as acting directly by the Yops action on the immune cells implicated in the specific immune responses. In this way, the inactivation of T cells leading to a reduction of the IFN-γ and TNF-α secretion inhibits the innate responses (Figure 2).

Interfering with the antigen presentation of DCs

Dendritic cells are in the interface between innate and adaptive immunity, playing a central role in development of both the immune responses. The main role of DCs is to capture the pathogens in peripheral tissues and to move into secondary lymphoid organs to present the antigen to naive T lymphocytes. The antigen presentation happens thanks to the ingestion and removal of pathogens within the phagosome followed by its presentation onto the major histocompatibility complex (MHC) molecules. The DCs activate the T cells by an MHC-specific manner [49], providing the required costimulatory signals. Moreover, the DCs contribute to the T lymphocytes activation by avoiding the suppression of regulatory T cells (CD4 and CD8) by production of IL-6 [50].

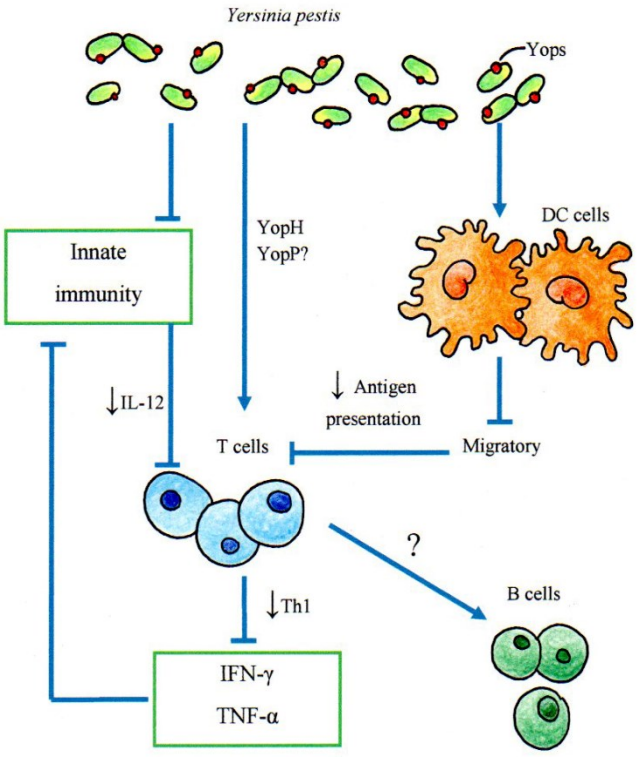
Numerous infectious agents are able to prevent the host defenses, for example compromising DC maturation, favoring DCs' apoptosis, or inhibiting cytokines' secretion. While *Y. enetrocolitica* is able to suppress the surface presentation of MHC class II and costimulatory molecules [51], *Y. pestis* causes the cytoskeleton rearrangement that paralysis DCs' movement [52], by the Yop's injection [53]. This mechanism deeply hamper the DCs' presentation of *Y. pestis* antigens to adaptive cells. The authors found, using Yop β-lactamase hybrids and fluorescent staining of live cells from plague-infected animals, that *Y. pestis* selects immune cells for injection. *In vivo,* macrophages, neutrophils and especially dendritic cells were injected most frequently, whereas B and T lymphocytes were rarely selected for injection. In this way, *Y. pestis* disables these cell populations to annihilate host immune responses during the infection [53].

Inhibition of the T-cell activation

Yersinia pestis is also able to affect directly the adaptive immunity by the suppression of T-lymphocytes' activation. The T3SS protein YopH has been previously demonstrated to be able to inhibit the adaptive immune response *in vitro* [54].

One study have shown that after a transient exposure to *Y. pseudotuberculosis*, T and B cells are impaired in their ability to be activated through their antigen receptors; specifically T cells are inhibited in their ability to produce cytokines (e.g. IL-2), and B cells are unable to upregulate surface expression of the costimulatory molecule, B7.2, in response to antigenic stimulation. The block of lymphocyte activation resulted from the inhibition of early phosphorylation events of the antigen receptor signaling complex. Using *Y. pseudotuberculosis* mutants, the authorsdemonstrated that the inhibitory effect in both T

Figure 2. Mechanisms of *Y. pestis*' resistance to the host adaptive immune response.



The plague bacilli are able to reduce the host adaptive immune response both influencing the cytokine induction (for example reducing IFN-γ and TNF-α) and acting directly by the Yops action on the immune cells involved in the specific immune responses.

cells and B cells is dependent on the production of Yop H [54].

Recently, Alonso and colleagues showed that YopH inhibits the activation of T lymphocytes by dephosphorylating the Lck tyrosine kinase at Tyr-394, resulting in a complete loss of its catalytic activity [55]. In another study, the same authors demonstrated that prolonged presence of YopH in primary T cells causes apoptosis, detected by annexin V binding, mitochondrial breakdown, caspase activation, and internucleosomal fragmentation. YopH also causes cell death when expressed in HeLa cells, and this cell death was inhibited by YopH-specific small molecule inhibitors. Cell death induced by YopH was also prevented by caspase inhibition or co-expression of Bcl-xL. They conclude that YopH not only paralyzes T cells acutely, but also ensures that they lose the ability to trigger apoptosis by mitochondrial pathway [56].

Moreover, in murine models, the YopP isolated from *Y. pseudotuberculosis* was able to inhibit the expansion of a CD8 T-cell response [57]. However, although this species is closely related to *Y. pestis*, its infection mechanisms can be different as demonstrated by the fact that *Y. pseudotuberculosis*, like *Y. enterocolitica,* usually provokes a chronic infection, while *Y. pestis* causes systemic infections [58]. For example, according to studies on *Y. enterocolitica,* T3SS protein YopJ can induce the programmed cell death of phagocytes, but this determinant is not injected by *Y. pestis* [59]. In addition, the studies about *Yersinia pestis* and host immune system were mostly focused on T3SS proteins. To better characterize the plague pathogenesis, more specific investigations aimed at understanding and elucidating the interaction between *Yersinia pestis* and immune systems should be conducted.

Yersinia Pestis-specific immune response

During *Y. pestis* infection, humoral and cellular immunity cooperate to provide a protection to the host. Antibodies produced by B-lymphocytes can directly neutralize the extracellular bacilli as well as support the cell-mediated immunity by favoring T-cell activation. Together, the cellular immune responses can aid humoral protection by eradicating intracellular *Y. pestis* sources.

The elucidation of the role of *Y. pestis*-specific immune response in the host will give important notions for the characterization of bacterial virulence and will also allow the production of more specific countermeasures.

Antibody-mediated defense against Y. pestis

Numerous proteins belonging to *Y. pestis* (see Table 1) are able to stimulate the production of specific antibodies by B lymphocytes**,** both in human and animal models. Moreover, to identify new immunogenic molecules or protective antigens it is possible to profile the antibody host's response, through proteomic technologies [60]. For example, by the antigenome technology, Yang's group has identified at least ten novel immunogenic proteins, such as YPO2090, YPO2091, YPO2102, YPO2112, YPO2118, YPO2131, YPO2190, YPMT1.12c, YPMT1.24c, and YPMT1.75c [61].

It is well known that naïve mice can be immunized by the injection of plague convalescent patients' serum, thus supporting the protective role of the antibody response against the *Y. pestis* infection. Moreover, subunit vaccines, created with the high immunogenic proteins F1 and LcrV, can supply protection in small animal models, with an antibody mediated mechanism [62-64]. Besides F1, and LcrV, other five proteins (YopD, YpkA, YscF, YadC, and OppA) are able to elicit a protective immune response in the host [65,66].

Although the efficacy of vaccines based on F1/LcrV subunits in small animals, at a Plague Vaccine Workshop sponsored by the Federal Drug Administration's Center for Biologics Evaluation and Research, USAMRIID presented impressive data from a series of primate vaccine trials [67]. The overall

Table 1. Immunogenic proteins of *Y. pestis.*

Protein	Role
LcRV	V antigen
YscF	Type III secretion apparatus component
YscC	Type III secretion apparatus component
YscJ	Type III secretion apparatus component
YscO	Type III secretion apparatus component
YscP	Type III secretion apparatus component
VirG	Targeting protein of the YscC complex
YopN	Type III membrane-bound Yop targeting protein
TyeA	Type III secretion and targeting protein
YopD	Type III targeting component
YopH	T3SS effector
YopE	T3SS effector
YopM	T3SS effector
YpkA	T3SS effector
YopK	Type III virulence determinant protein
OppA	Oligopeptide periplasmic binding protein
Pla	Coagulase/fibrinolysin precursor
PsaA	pH6 antigen
LPS	Lipopolysaccharides
YadC	Outer member protein
F1	F1 capsule antigen

*

conclusion was that F1/V-based vaccines provide cynomolgus macaques with significant protection against aerosolized *Yp* challenge, but fail to adequately protect African green monkeys. Presently, we lack a satisfactory explanation for the variable efficacy of F1/V-based vaccines in non-human primates. As such, there is substantial concern that F1/V vaccines may fail to protect humans against weaponized plague [67]. In other words, antibodies do not seem to effectively protect against pneumonic plague.

Most recently Del Prete and collaborators [68] have described, using *in vitro* models, the effects of *Yersinia pestis* recombinant protein rF1, rV, and rF1-V on human cells of adaptive immunity especially of B cells.

In detail, using ELISA with native F1 or rF1 (recombinant F1) to detect anti-F1 IgG antibodies in *Y. pestis*-primed individuals, they showed that antibody response to F1 was detectable in patients tested within 20 months from the plague, vanishing thereafter. In the same sera, reactivity to rF1 was slightly, but consistently, higher than to native F1, suggesting that rF1 is suitable for diagnostic procedures. In comparison to rF1, rF1-V was not equally well recognized by sera of *Y. pestis*-primed subjects. The lower efficiency of rF1-V for antibody binding may be due to poor recognition of its V portion and to reduction of B-cell epitopes in the F1 portion due to the fusion protein conformation.

Cellular immune defenses to Y. pestis

Increasingly proofs display the importance of T cell responses in the struggle against *Y. pestis* infection [69,70].

Type 1 immune reactions, usually implemented toward cancer cells or cells infected by intracellular pathogens, are related to a milieu with cytotoxic functions including enhanced NK, Th1, and $CD8^+$ T cell activities; IFN-γ, TNF-α, and nitric oxide synthase 2, seem to be fundamental also in the fight against pulmonary *Y. pestis* infection. In fact, Philipovskiy and Smiley demonstrated that vaccination with live *Y. pestis* primes Th1 and $CD8^+$ T cells that respond to *Y. pestis* strains lacking the capacity to express F1, LcrV, and all pCD1/pPCP-encoded proteins, suggesting that protective T cells recognize antigens different from B cell's antibodies. These observations strongly suggest that development of pneumonic plague vaccines should strive to prime both CD4 and CD8 T cells [71]. Furthermore, the transfer of the *Y. pestis*-primed T cells to naive μ-MT mice protects against fatal intranasal *Y. pestis* challenge, suggesting that cellular immunity, in the absence of antibody, can protect the animal against pulmonary plague [70]. Then, it is clear that vaccines, to be more efficient, should contain different antigens able to elicit both antibody- and T-cell-mediated immunity.

Recently, there has been considerable interest in using transgenic plants to generate compounds for medical and veterinary use [72,73]. The use of a unique vector for robust expression of *Y. pestis* rF1, rV, and rF1-V fusion proteins in leaves of *Nicotiana benthamiana* was described [74,75]. The plant-derived *Yersinia pestis* antigens effectively protected guinea pigs against aerosol challenge with *Yersinia pestis* at doses 100% lethal to unvaccinated animals controls [76].

In recent times, we have described the *in vitro* effects of *Y. pestis* rF1, rV, and rF1-V generated in *N. benthamiana* on human cells of the innate and adaptive immunity [68].

This study showed that recombinant plant-derived rF1, rV and rF1-V are TLR2 agonists and importantly, they significantly increase IL-6 and at a lower degree CXCL-8 production by human monocytes, without affecting TNF-α IL-12, IL-10, IL-1β, and CXCL10 production.

The data suggest that plant-derived rF1, rV and rF1-V are poorly reactogenic on human cells of the innate immunity. No induction of pro-inflammatory cytokines, low induction of IL-8 and upregulation of IL-6 represent important features of rF1-V in view of its use as candidate vaccine for oral immunization, since no inflammation nor neutrophilia is expected in the gut, while upregulation of IL-6 may be regarded as a promise of prolonged plasma cell survival and antibody response upon vaccination.

The V-antigen (LcrV) was described as the major virulence marker of *Y. pestis* [77]. In mice, V antigen is an immunomodulator (TNF-α and IFN-γ down-regulation and IL-10 induction) both *in vivo* and *in vitro* [78-84]. Such effects would depend on TLR2 stimulation [85]. In a recent study, the agonistic interaction of rV with TLR2 was confirmed. Also rF1 and rF1-V were TLR2 agonists in the same range of relatively high protein concentration. Though rV was slightly more reactogenic on human monocytes than rF1, nor up-regulation of IL-10 was detected, nor down-regulation of pro-inflammatory cytokines. Whether the lack of rV protein-induced IL-10 increase depend on the source of responder cells (murine vs human) or on recombinant V proteins (bacterial vs plant), remains unclear.

In any case, the lack of IL-10 induction in human cells by plant-derived rV and rF1-V represents a positive feature of these candidates for oral vaccination.

Moreover, the study [65] showed that rF1, rV, and rF1-V are recognized by memory T-cells and by serum antibodies of a number of patients who recently healed from plague.

In detail, in the three (of the 20 subjects) tested within 20-40 days from disease, no proliferation of T cells was detectable, though they had already converted to seroposivite for anti-F1 antibodies. Likewise, apart from one donor who showed a poor T-cell response to F1, rF1 and rF1-V, but not to rV antigen, other 5 donors tested after 26 months or more from diagnosis showed no proliferation of their circulating T cells to native or recombinant *Y. pestis* antigens. Among other 11 *Y. pestis*-primed donors tested between 2 and 20 months from diagnosis, 7 showed T-cell proliferation to either native F1 or rF1 and rF1-V at the highest concentration (10 μg/ml), but not to lower antigen doses.

In six of of these eleven donors, T-cell proliferation to rV antigen was also detectable, though lower than to native F1, rF1 or rF1-V. A significant T-cell proliferation to native F1, rF1, and rF1-V, but not to rV antigen, was observed also in the healthy exposed donor. These data suggest that in the peripheral blood of subjects who got plague, the proportion of T cells specific for native F1 or rF1, rV, and rF1-V was quite low, and needed at least one month to become detectable in proliferation assays. The need of a relatively high antigen concentration to achieve significant mitogenic indexes argues in favour of this explanation. The data also suggest that in *Y. pestis*-primed donors, the presence of circulating *Y. pestis* antigen-specific T cells is relatively short-term, vanishing after 2 years. Since the analysis of responsiveness by T cells derived from other sources, such as lymph nodes, was not feasible, the reason why T-cell memory for *Y. pestis* antigens is short lasting in comparison to the memory against other pathogens, remains unanswered.

T-cell responsiveness to V antigen was never dissociated from that against F1, but T-cell proliferation to V was consistently lower than to F1. The most simple explanation is that during *Yersinia pestis'* infection, priming of T-cell response to V antigen was less powerful than to F1. This would have resulted in lower proportions of recirculating V-specific memory T cells and hence lower proliferative responses in *in vitro* models.

Strategies for efficient Y. pestis vaccination

All the aspects analyzed in previous sections, together with the possibility to elicit antibody responses resembling those of *Y. pestis*-infected subjects and the high protection of guinea pigs from the pulmonary disease, allow to foresee good perspectives for these plant-derived antigens as oral vaccine for prevention of plague.

However, to develop an efficient vaccine, the identification of the different antigens able to elicit a protective T cell response, is crucial. Then, Yang and collaborators [85] have used *in silico* analysis and an *in vitro* IFN-γ assay to identify novel *Y. pestis* potential T-cell antigens. In this study, 261 genes from *Y. pestis* were selected on the basis of bioinformatics analysis and previous research results for expression in *Escherichia coli* BL21(DE3). After purification, 101 proteins were qualified for examination of their abilities to induce the production of IFN-γ in mice immunized with live vaccine EV76 by enzyme-linked immunospot assay. Thirty-four proteins were found to stimulate strong T-cell responses. The protective efficiencies for 24 of them were preliminarily evaluated using a mouse plague model.

In addition to LcrV, nine proteins (YPO0606, YPO1914, YPO0612, YPO3119, YPO3047, YPO1377, YPCD1.05c, YPO0420, and YPO3720) may provide partial protection against challenge with a low dose (20 times the 50% lethal dose (20× LD (50)) of *Yersinia pestis*, but only YPO0606 could partially protect mice from infection with *Yersinia pestis* at a higher challenge dosage (200× LD(50)). These proteins would be the potential components for *Y. pestis* vaccine development.

Future Prospects

The elucidation of the interactions between *Yersinia pestis* and host is mandatory for the understanding of the different aspects of the disease's pathogenesis and for the planning and the development of successful countermeasures.

Yersinia pestis is the infamous agent responsible of plague and represent a bioweapon impending public health. To the development of new and more efficient vaccines, it is important to clarify the effector immune responses that the host can implements against the plague's bacilli. Both components (B and T lymphocytes) of the adaptive immune response contribute to protect the host from *Yersinia pestis*. However, our current knowledge about the adaptive immune mechanisms elicited during plague infection is still limited. Today, the technological advancement, in

particular the omics science's field (such as immunomics, genomics, proteomics, metabolomics, ecc.) can offer us new important information for the expansion of more effective and innovative treatments against *Yersinia pestis'* infection.

Memorial Addendum

This manuscript is dedicated to the memory of Professor Gianfranco Del Prete, who recently passed away. I and my group personally have to thank the Professor who, with his professional rigor, gave us all the basis for a right and correct scientific research.

In addition, he gave me the passion and dedication in the study of the fascinating galaxy of immunology.

The studies of the Professor Del Prete provided significant and important contributions in various fields of basic and clinical immunology, principally in the different aspects of the immune response pathophysiology; the host adaptive immune response to pathogens; the pathogenetic characteristics of organ-specific autoimmune diseases; and finally the allergy pathogenesis.

Recently, the Professor Del Prete had obtained strong evidence for a potential use of plant-derived antigens as oral vaccine for the prevention of plague and so the correlated comorbidity.

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